Tamoxifen Induces Hypoxia in MCF-7 Xenografts

Sydney M. Evans, Cameron J. Koch, Kristine M. Laughlin, W. Timothy Jenkins, Thomas Van Winkle, and David F. Wilson

ABSTRACT

Tamoxifen is widely used as an adjunct therapy for breast cancer. We hypothesized that hypoxia develops in tumors as a result of tamoxifen treatment because tamoxifen has been reported to be angiostatic and thrombogenic. MCF-7 breast tumors were grown under estrogen stimulation in 4-6-week-old CD-1 nu/nu female mice. When the tumors were approximately 5 mm in diameter, 17β-estradiol pellets were replaced with either placebo or tamoxifen-containing pellets. Two days later, tissue oxygenation was measured using immunohistochemical detection of binding of the 2-nitroimidazole EF5. Intravascular oxygen partial pressures were measured noninvasively by oxygen-dependent quenching of phosphorescence of an injected dye that is excited by light pulses. Tamoxifen treatment increased hypoxia in the tumors, as measured by EF5 binding (P = 0.01 by Mann-Whitney test). This observation was not dependent on the presence of tamoxifen-induced necrosis. Intravascular oxygen partial pressures were lower in tumors relative to surrounding normal tissue in tamoxifen-treated tumors as compared to placebo-treated tumors. In vitro, tamoxifen did not modify the oxygen-dependent metabolism of EF5, indicating that the increased EF5 binding in tamoxifen-treated tumors reflects a physiological decrease in tissue oxygenation. The clinical significance of these observations is discussed in the context of the sequencing of tamoxifen with other therapies, and in light of recent data suggesting that hypoxia may be associated with genetic changes resulting in a more aggressive tumor phenotype.

INTRODUCTION

Currently available treatment options for breast cancer include surgery, chemotherapy, hormonal manipulation, and radiation therapy (for a review, see Ref. 1). One of the most complex issues in combined modality breast cancer therapy is the sequencing of these modalities. At some institutions, patients with breast cancer receive tamoxifen concurrent with radiotherapy (2). We were concerned that tamoxifen’s effect on tumor vasculature (and subsequent tissue oxygenation) could negatively affect radiation response; this was based on the observations that tamoxifen has been shown to cause tumor necrosis and regression via inhibition of angiogenesis and endothelial cell growth in MCF-7 breast tumor xenografts (3, 4). Also, clinically significant deep vein thrombosis has been reported to occur in a small percentage of patients on tamoxifen therapy (5, 6). As a result of these vascular changes in tamoxifen-treated tumors (e.g., thrombosis and inhibition of angiogenesis), cells may undergo starvation, anoxia, and death. However, adjacent to areas of necrosis, there are likely to be regions that are hypoxic and contain viable cells. The presence of hypoxic, clonogenic tumor cells is known to be an important cause of radiation treatment resistance in cellular systems, animal models, and human patients (7-9). The presence of hypoxia is associated with poor therapeutic response in women with cervical cancer (10), head and neck cancer (11, 12), and soft tissue sarcoma metastasis in patients treated with hyperthermia, radiation, and surgery (13). Hypoxia has also been shown to induce molecular changes in cells, including those that may confer a more malignant phenotype (14, 15).

Over the last 5 years, there has been a marked improvement in the ability to measure tissue oxygen partial pressures. Although there are many techniques available (16), three techniques are currently being used clinically: (a) the Eppendorf needle electrode (11, 13); (b) the comet assay (17); and (c) nitroimidazole binding techniques (18). Nitroimidazole binding techniques are unique in that they can measure hypoxia over the smallest distances expected to have oxygen gradients (e.g., cell-to-cell distances) and have a positive signal in the absence of oxygen. Identification of the binding sites can be accomplished using several detection methods including positron emission tomography (19), nuclear medicine (20), and antibody-based methods (21). Monoclonal antibody detection of the 2-nitroimidazole EF5 has been described using immunohistochemical as well as flow cytometric techniques (22, 23). Binding of EF5 has been shown to be relatively independent of cell or tissue type (22), is unaffected by nonoxygen-containing substances (21), and was found to correlate with several therapeutically relevant end points (8, 24, 25).

Only one oxygen-measuring technique, PLI,3 is capable of monitoring in vivo oxygenation in real time. PLI is an optical method that relies on the oxygen dependence of phosphorescence as described by the Stern-Volmer relationship (26). A phosphorescent dye (27) is administered i.v., and the area of interest (e.g., the tumor region) is exposed to excitation light pulses. Oxygen is the only compound in normal blood that quenches phosphorescence; the resulting analyses give unambiguous measurements of intravascular oxygen pressure (28). These measurements have been shown to correlate with radiation sensitivity in s.c. 9L tumors (29).

Human breast cancer is characterized by variable sensitivity to growth modification by hormones and their antagonists (30), variable radiation response (2), and distinctly heterogeneous presence of hypoxia (31). The interaction of these properties has not been previously studied but may affect breast cancer treatment and management. We have investigated the interactions between hormonal modifications and hypoxia in the MCF-7 tumor, an estrogen-receptor-positive human breast carcinoma grown as a xenograft in athymic mice (32). Based on data obtained from immunohistochemical detection of the binding of EF5 and the PLI technique, we conclude that tamoxifen induces hypoxia in MCF-7 tumors.

MATERIALS AND METHODS

Animals and Tumors. All animal studies were performed under regulations provided by the University of Pennsylvania Institutional Animal Care and Use Committee. MCF-7 tumors were implanted s.c. in either the flank or the caudal mammary fat pad (orthotopic) of 4–6-week-old female, intact athymic CD-1 mice. At the time of injection of 5 million MCF-7 cells suspended in Matrigel (10 mg/ml; Collaborative Research), a pellet of 17β-estradiol (0.72 mg/pellet) was implanted beneath the back skin. When the tumor was approximately 5 mm in diameter (range, 4–8 mm), the estrogen source was removed, and either a tamoxifen-containing pellet (5 mg released over 60 days with a

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3 The abbreviation used is: PLI, phosphorescence lifetime imaging.
blood level of 3–4 ng/ml or a placebo pellet [all pellets from Innovative Research of America (Toledo, OH)] was implanted. Five mice were treated with a placebo pellet, and seven mice were treated with a tamoxifen-containing pellet. Two days after the implantation of pellets, the tumors were studied for the presence of hypoxia using immunohistochemical detection of nitroimidazole (EF5) binding and/or PLI. Tumors from all 12 mice were studied for EF5 binding. Three placebo-treated mice and four tamoxifen-treated mice were studied with PLI.

PLI. Green 2W phosphorescent probe (27) in physiological saline [0.3 ml (pH 7.4)] was injected i.v. via the tail vein within 1 h of study. All PLI studies were performed in orthotopically implanted tumors. The tissue was observed through a Wild-Leitz macrozoom microscope using an intensified Gen. II CCD camera (26). The phosphorescence lifetime was calculated by best fit to a single exponential at each pixel of the image array. The measured decay constants were substituted into the Stern-Volmer equation, and the oxygen pressure in each region of interest was calculated using a lifetime in the absence of oxygen of 340 μs and a quenching constant of 250 torr/s. To quantitate the difference in oxygen pressure between the tumor and adjacent normal tissue, two straight, approximately perpendicular lines passing through the tumor area were drawn across the oxygen pressure maps. The oxygen pressure at each point along the line was then plotted against the position along the line.

**EF5-based Hypoxia Studies.** A pentafluorinated derivative of etanidazole was synthesized by the National Cancer Institute (Dr. R. Vishnunavajala) and is referred to as EF5 in this manuscript. Labeled EF5 (2-18O; position; 43 μCi/mg) was synthesized by Dr. M. Tracy and colleagues (Stanford Research International, Palo Alto, CA). The monoclonal antibodies used in the present study were produced as described previously (21) and were from a single hybridoma clone designated as ELK3-51. The slides were conjugated with the green-excited, orange-emitting fluorescent dye Cy3.

**Cell Culture and EF5 Binding Calibration Control Studies in Vitro.** Estrogen receptor-positive MCF-7 breast cancer cells were obtained from the American Tissue Type Collection (Washington, DC). Strock cultures of MCF-7 cells were routinely maintained in DMEM supplemented with 10% FCS and antibiotics (penicillin and streptomycin) as a standard medium. Cultures were maintained in a 100% humidified atmosphere of 5% air + 5% CO2 at 37°C. Two days before a calibration experiment, standard medium was replaced with media containing 1 μM tamoxifen in 0.1% ethanol or 0.1% ethanol for control cells. On the day before an experiment, cells were trypsinized, and 2.5 × 10^6 cells were plated onto the central areas of 50-mm glass Petri dishes, followed by incubation at 37°C for approximately 16 h (33). The dishes were removed from the incubator and cooled, and their media were replaced with EF5-containing media, as required. Dishes were exposed to the desired oxygen concentration (<0.01, 0.25, 1, and 4%; Ref. 22) and 100 μM EF5 for 3-5 h to simulate the in vivo drug exposure in a mouse (approximately 0.25 μM/h). The adherent cells were rinsed and removed from the dishes, and the cell number was determined with a particle counter ( Coulter). B0 was determined using standard liquid scintillation techniques (for radioactive drug) or flow cytometry techniques (for nonradioactive drug; Ref. 22).

**Determination of Maximum Binding (Bmax) of MCF-7 Tumor Tissue in Vitro.** To determine the maximum EF5 binding (Bmax) of MCF-7 tumor tissue, portions of freshly removed tumor were diced into cubes approximately 1–2 mm/side. Tissue cubes were incubated in stirred suspension with 200 μM EF5 and a gas phase containing <0.01% oxygen for 2 h at 37°C and then frozen at −80°C. Frozen 14-μm sections were collected and stained with Cy3-conjugated antibodies (ELK3-51) as described previously (23) and briefly discussed below. For such samples maintained under anoxic conditions, maximal binding at the tissue surface would be expected (34). This bright binding was quantitated and defined the Bmax for the MCF-7 tumor. These data were used to interpret the significance of the median EF5 binding obtained as a result of the in vivo EF5 exposure.

**In Vivo Studies.** In vivo EF5 binding studies were performed in mice bearing MCF-7 tumors located either in the flank or orthotopically in the caudal mammary fat pad. The EF5 binding results obtained in both sites were the same; therefore, the data were combined. Tumor-bearing mice were given two injections each of 10 mm EF5 prepared in 0.9% saline (the volume of administration of each dose, in milliliters, was 0.01 × the animal’s weight in grams). The first injection was given i.v., and the second injection was given i.p. 15 min later. For those animals receiving a PLI study, 2 h after EF5 administration, the animal was anesthetized with an i.p. xylazine (1.3 mg/kg) and ketamine (140 mg/kg). Green 2W was injected i.v., and the mouse was positioned for PLI imaging. After this imaging study, the tumors were removed and frozen for sectioning. Those animals who were not studied with PLI were euthanized 3 h after EF5 injection, at which time the tumor was removed and frozen.

**Tumor tissue sections were cut, fixed, blocked, and stained as described previously (23). Briefly, 14-μm sections were saved at 1-mm intervals, fixed for 1 h at 4°C in PBS with 4% paraformaldehyde, and blocked against nonspecific binding at 4°C overnight. The slides were then rinsed, and 75 μg/ml Cy3-conjugated ELK3-51 antibody was added for 4–6 h at 4°C. The slides were rinsed and stored in PBS with 1% paraformaldehyde until photographed, usually within 3 days.**

The tissue sections were analyzed by measuring the fluorescence of the Cy3-conjugated anti-EF5 monoclonal antibodies using a Nikon fluorescence microscope fitted with a sensitive digital camera (Xillix, Vancouver, British Columbia, Canada). Multiple whole tumor sections were imaged. After photography of EF5 binding, slides were stained with H&E and evaluated as described below.

**EF5 Image Analysis.** To make the fluorescence evaluation as quantitative as possible and to allow comparison from sample to sample, day-to-day variations in the lamp intensity were accounted for by filling the well of a hemocytometer with a reference concentration of Cy3 dye (a concentration with an absorbance of 0.42 at 549 nm) and then imaging the hemocytometer well while focusing on the grid lines. Variations in the fluorescence intensity of the reference dye (typically a factor of up to 4 over the life of a quartz-halogen bulb) were then used to calculate the intensities of the section images. The section images were photographed so that the maximum pixel intensities were within the linear range of the camera (0–255). Two camera variables are set by the software: (a) the camera sensitivity (S; varying from 1/256 to 1/4096 in factors of 2); and (b) the camera exposure time (T; varying from less than 100 to 10,000 ms). Therefore, each pixel intensity is corrected by the factor (S/4096) × (10,000/T). The corrected pixel intensities of each image are divided by the median of the corrected pixel intensity of the hemocytometer calibration standard to determine the final absolute fluorescence intensities.

**Histopathological Analyses.** Slides containing 14-μm frozen tissue sections that had previously been photographed and analyzed for EF5 binding were air-dried, stained with H&E, and coverslipped. A board-certified veterinary pathologist (T. V. W.) reviewed the slides without prior knowledge of whether the tumors had been treated with tamoxifen. Necrotic cells were defined as those that were shrunken or fragmented with hypereosinophilic cytoplasm and pyknotic or fragmented nuclei. Necrosis was graded on a 0–5 scale: 0, no necrosis; 1, rare necrotic cells; 2, scattered clusters of necrotic cells (<30% of cells); 3, groups of necrotic cells in most tumor cords (<30% of cells); 4–75% cells necrotic; and 5, most or all cells necrotic. Infiltration by immune cells was noted as either peripherally located or scattered.

**Statistical Analyses.** Mann-Whitney test statistical analyses were applied to the median EF5 binding of tumors treated with either placebo or tamoxifen-implanted pellets. The level of significance was defined as P < 0.05.
Assessment of Hypoxia in Tamoxifen-treated MCF-7 Xenografts. Fig. 3 demonstrates that placebo-treated tumors generally bound little EF5 and were considered to be well oxygenated. After 2 days of tamoxifen therapy, the median EF5 binding was substantially increased (P = 0.01 by Mann-Whitney test). Fig. 4, A and C, provides an example of the patterns of EF5 binding in one placebo-treated and one tamoxifen-treated tumor. Several small regions of binding are present in the center of the placebo-treated tumor. In contrast, EF5 binding is seen diffusely through most of the tamoxifen-treated tumor. After photography of the EF5 fluorescent images, slides were dried and stained with H&E. Two to 5 sections of 10 tumors were analyzed for the presence of necrosis and cellular infiltrates. Tumor tissue was organized into cords, clusters, and acini composed of polyhedral to cuboidal cells with large basophilic nuclei and eosinophilic cytoplasm. Microscopic necrosis was usually located in the center of the cords, clusters, or acini. Macroscopically, there was a tissue-free region with adjacent neutrophils in the center of two of six tamoxifen-treated tumors. This region was interpreted to represent central necrosis that had washed off the slide in the processing. Such a region was not present in any of the placebo-treated tumors. The median amount of necrosis identified in the placebo-treated tumors did not differ from that in the tamoxifen-treated tumors when the analysis was performed to exclude the two tumors with central tumor-free regions or to include all tumors; the median necrosis score was 2.00 (n = 33; range, 0—3.00) in control tumors and 2.00 (n = 33; range, 0—3.00) in tamoxifen-treated tumors, including those with a central tumor-free region. Immune and/or inflammatory cell infiltrates, presumably B lymphocytes, macrophages, and NK cells, were present in both treatment groups (data not shown).

In three placebo-treated and four tamoxifen-treated animals, PLI studies to evaluate intravascular oxygen content were performed immediately before tumor removal and freezing for immunohistochemical analyses. Fig. 4 presents one example of the comparison between binding of tumors removed from placebo versus tamoxifen-treated mice, inferring that there is no tamoxifen-dependent induction or inhibition of binding. In situ binding (Figs. 2 and 3) is substantially less than the maximum EF5 binding in the tissue cubes, suggesting that tumor oxygenation is intermediate between physiological values (4% oxygen) and severe hypoxia (<0.01% oxygen; Refs. 35 and 36).

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Fig. 4. A and C, the patterns of EF5 binding in one exemplary placebo-treated (A) and one tamoxifen-treated (C) tumor. Several small regions of binding are present through the center of the placebo-treated tumor. In contrast, EF5 binding is seen diffusely through most of the tamoxifen-treated tumor, with patterns suggestive of tumor cords. B and D, intravascular oxygen tension based on PLI. In the placebo-treated tumor, almost all of the intravascular oxygen levels in the tumor region are at the same level as adjacent normal tissue. The tumor region was marked on the mouse’s skin with an indelible marker; this effectively blocked phosphorescence and created the red-black semicircular artifact. In contrast, the tamoxifen-treated tumor is well delineated from the adjacent normal tissue in the PLI studies by virtue of its lower intravascular oxygen levels.

DISCUSSION

The impetus to study the effect of tamoxifen on tumor tissue oxygenation came from reports that this agent can both increase the incidence of vascular thromboses in humans (37) and inhibit angiogenesis via endothelial growth in several assay systems (3, 37). We studied tumors after 48 h of tamoxifen therapy, based on magnetic resonance spectroscopic observations that central tumor necrosis could be seen after as little as 12–48 h of tamoxifen therapy in the MCF-7 tumor model (4). Our histological findings of central tissue-free regions in two tamoxifen-treated tumors are consistent with these magnetic resonance spectroscopic observations (4), because necrotic tissues are often lost from the slides during the fixation of frozen sections. The presence of necrosis is a negative prognostic factor in primary breast cancer and may increase the risk for local recurrence (38). It has been suggested that necrosis heralds regions of low tumor oxygenation (38). We hypothesized that tamoxifen therapy could result in the development of hypoxic clonogenic cells in association with regions of necrosis. In several of our tumors, hypoxia occurred in the absence of tamoxifen-induced necrosis. This implies that necrosis is not required for the development of hypoxia and suggests that intermediate hypoxia may predominate in these instances. Recent studies suggest that intermediate rather than severe hypoxia may be most important in radiation resistance (24, 35, 36). Perhaps of most concern is the observation that hypoxic tumor cells undergo genetic changes making them more phenotypically aggressive (14) and that hypoxic tumors are more likely to metastasize (13).

We have used two independent techniques to measure the effect of

5 S. M. Evans and C. J. Koch, unpublished observations.
Fig. 5. Line intensity profiles of the tumor oxygenation as measured by PLI. Two straight lines were drawn approximately at right angles across oxygen pressure maps of the tumor and surrounding tissues. The lines extended from normal tissue through the center of the tumor area and into normal tissue on the other side of the tumor. The oxygen pressures were then plotted for each point along the line against its position within that line. The oxygen pressures in the tumor can be compared with those from normal tissue on either side of the tumor, providing a control for mouse-to-mouse differences in tissue oxygenation arising from differences in anesthesia, respiratory rate, and so forth. In graph 0206-GK-5P, several extremely high readings (>40 torr) are seen in positions 5–8 and 15–18. These are artifactual and correspond to markings placed on the skin surface to delineate the tumor location (see Fig. 4D). In other tumors, skin markings were not used. The flat solid and dotted lines in each figure represent the tumor region corresponding to the oxygen pressure data.
tamofoxen on the development of hypoxia in MCF-7 tumors in nude mice. EF5 binding has been shown to correlate with oxygen partial pressure in cellular (22), multicellular (8), and animal model systems (23). EF5 binding has also been shown to correlate to radiation response in KHT mouse sarcoma (39) and 9L rat glioma (24). In each of these rodent cell lines studied, a calibration curve was performed to confirm the relationship between EF5 binding and oxygen partial pressure. These curves demonstrate the wide dynamic range of EF5 binding relative to oxygen partial pressure. Criticisms of the nitroimidazole binding techniques for the diagnosis of hypoxia in tamoxifen-treated tumors include the possibility of oxygen-independent increases in EF5 binding. This possibility is of concern based on a recent observation that antiestrogens, including tamofoxen, up-regulate the quinone reductase gene (40). The mechanism of EF5 binding in cells, and the basis of our assay system, is that the drug is reduced and subsequently binds to cellular protein sulfhydryls. The reaction is maximized in the absence of oxygen and is inhibited in its presence, but the specific nitroreductase involved has not been identified. Therefore, we performed the ex vivo-in vitro tissue cube calibration study, expecting that if tamofoxen was up-regulating one of the critical nitroreductases, there would be increased EF5 binding in the tamoxifen-treated tissue compared to the placebo-treated tissue. The absence of significant differences in EF5 binding, as well as the data provided by the Pli technique, supports our conclusion that tamofoxen treatment is causing physiological hypoxia in these tumors.

Oxygen levels within the tissue are a balance between delivery (flow and oxygen content of the blood) and oxygen consumption. Thus, any decrease in tissue oxygenation can arise from either a decrease in delivery or an increase in consumption. In placebo-treated tumors, the oxygen pressures within the tumor were similar to those in the surrounding tissue, whereas after tamofoxen treatment, the oxygen pressures in the tumors were substantially below those of the surrounding tissue. This could arise because tamoxifen treatment either decreased oxygen delivery to the tumor, possibly by increasing vascular resistance and/or altering tumor angiogenesis, or because it increased cellular oxygen consumption within the tumors, possibly by receptor-mediated alteration in the metabolic rates. Preliminary in vitro studies suggest that tamofoxen does not change cellular respiration (data not shown). We are investigating other mechanisms whereby tamoxifen would affect tumor oxygenation, including the determination of whether these effects are estrogen receptor-dependent. It is known that tamoxifen’s antiangiogenic effects (and that of other partial antiestrogens such as nafloxidine and clomiphene) are not modulated via the estrogen receptor but by a direct inhibition of endothelial cell growth factors such as vascular endothelial growth factor and basic fibroblast growth factor (41, 42).

Several recent reports suggest a correlation between the metabolic milieu and tumor metastasis. In an elegant study using quantitative bioluminescence imaging, high tissue concentrations of lactate in primary human head and neck cancer were correlated with metastatic spread (43). A recent report associates the presence of tumor hypoxia with metastasis in patients with soft tissue sarcomas (13). Needle electrode measurements were performed in 22 patients with high-grade sarcomas; the 18-month actuarial disease-free survival was 70% versus 35% in patients with high versus low Po2 values. The site of tumor recurrence was lung metastasis in the eight treatment failures. In another study, the Eppendorf needle electrode was used to measure presurgical oxygenation of cervix tumors in 47 women; the authors found that Fédération Internationale des Gynécologues et Obstétristes staging and tumor oxygenation were the most important independent factors for disease-free and overall survival (10). These clinical observations are paralleled by reports that hypoxia can cause genotypic and phenotypic changes (14, 15), rendering the tumors more aggressive. At this time, there is no direct evidence that tamofoxen induces hypoxia in human breast cancer. Indeed, the clinical experiences of tamoxifen are well known. It has been demonstrated that adjuvant administration of tamofoxen to women with estrogen receptor-positive breast cancer results in an improved disease-free survival and overall survival and a reduction in the development of contralateral breast cancer (44, 45). However, recent evidence from a large randomized trial suggests that in selected patients, the administration of tamoxifen beyond 5 years leads to decreased disease-free and distant disease-free survival (44). Studies on the cause of the therapeutically significant implication of our findings is the relationship of tamofoxen use to radiation therapy. If the induction of hypoxia by tamofoxen is identified as a significant clinical problem, it would be necessary to rethink the approach to multimodality breast cancer therapy. Studies to address this question are under way in our laboratory.

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REFERENCES


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